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DEVELOPMENTAL BIOLOGY

Developmental Biology 262 (2003) 335–349

www.elsevier.com/locate/ydbio

Ventral dominance governs sequential patterns of gene expression across the dorsal–ventral axis of the neuroectoderm in the *Drosophila* embryo

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Received for publication 19 November 2002, revised 12 June 2003, accepted 13 June 2003

Abstract

A nuclear concentration gradient of the maternal transcription factor Dorsal establishes three tissues across the dorsal–ventral axis of precellular *Drosophila* embryos: mesoderm, neuroectoderm, and dorsal ectoderm. Subsequent interactions among Dorsal target genes subdivide the mesoderm and dorsal ectoderm. Here we investigate the subdivision of the neuroectoderm by three conserved homeobox genes, *ventral nervous system defective (vnd)*, *intermediate neuroblasts defective (ind)*, and *muscle segment homeobox (msh)*. These genes divide the ventral nerve cord into three columns along the dorsal–ventral axis. Sequential patterns of *vnd*, *ind*, and *msh* expression are established prior to gastrulation and evidence is presented that these genes respond to distinct thresholds of the Dorsal gradient. Maintenance of these patterns depends on cross-regulatory interactions, whereby genes expressed in ventral regions repress those expressed in more dorsal regions. This "ventral dominance" includes regulatory genes that are expressed in the mesectoderm and mesoderm. At least some of these regulatory interactions are direct. For example, the misexpression of *vnd* in transgenic embryos represses *ind* and *msh*, and the addition of Vnd binding sites to a heterologous enhancer is sufficient to mediate repression. The N-terminal domain of Vnd contains a putative eh1 repression domain that binds Groucho in vitro. Mutations in this domain diminish Groucho binding and also attenuate repression in vivo. We discuss the significance of ventral dominance with respect to the patterning of the vertebrate neural tube, and compare it with the previously observed phenomenon of posterior prevalence, which governs sequential patterns of Hox gene expression across the anterior–posterior axis of metazoan embryos.

Introduction

Dorsal is a maternal Rel-containing transcription factor that is ubiquitously distributed throughout the cytoplasm of the unfertilized egg. Upon fertilization, a serine protease cascade results in asymmetric nuclear translocation of Dorsal, generating a broad dorsal–ventral gradient (Drier and Steward, 1997). This gradient establishes three embryonic tissues by regulating target genes in a concentration dependent manner through high- and low-affinity Dorsal binding sites (Stathopoulos and Levine, 2002). The ventral 18–20 nuclei give rise to the mesoderm, responding to the highest levels of the Dorsal gradient. The 30–32 nuclei on the dorsal side of the embryo with no nuclear Dorsal protein are fated to become dorsal ectoderm. The remaining nuclei in lateral regions, ~14–16 nuclei on either side of the ventral mesoderm, form the neuroectoderm. Interactions among Dorsal target genes further subdivide each of these tissues into multiple cell types.

Subdivision of the Dorsal ectoderm into amnioserosa and dorsal epidermis depends on a Dpp activity gradient (Ferguson and Anderson, 1992; Wharton et al., 1993). This gradient is formed by interactions between different Dorsal target genes, *short gastrulation (sog)* (Francois et al., 1994; Zusman et al., 1988) and *decapentaplegic (dpp)*. *sog* is activated by the low levels of Dorsal found throughout the lateral neuroectoderm. In contrast, the Dorsal gradient represses the transcription of *dpp*, restricting Dpp signaling to

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doi:10.1016/S0012-1606(03)00395-6

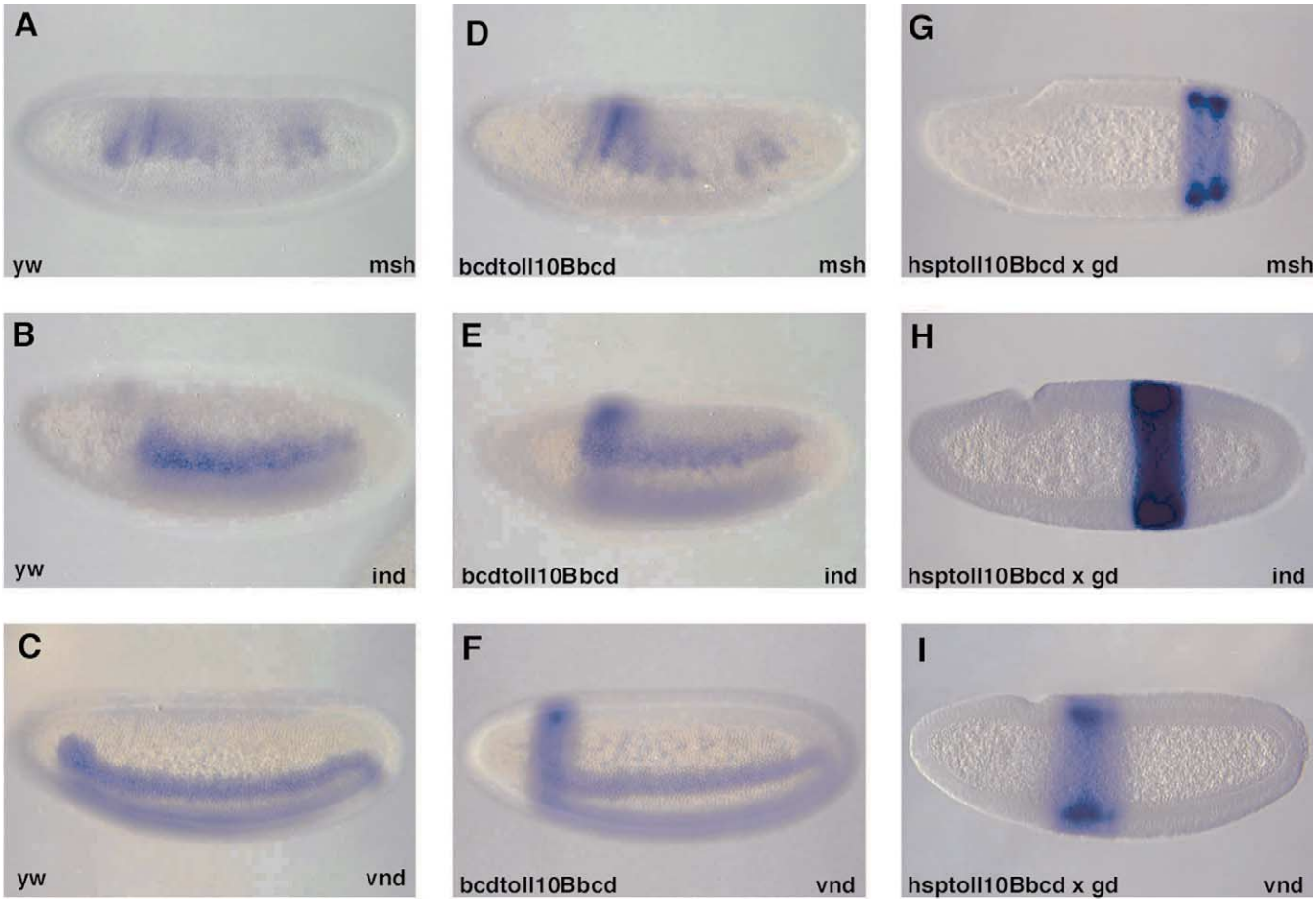

Fig. 1. Expression of *vnd, ind,* and *msh* in wild-type (A–C) and transgenic (E–I) backgrounds. Embryos were hybridized with dioxygenin labeled probes and photographed with anterior to the left and dorsal to the top. (A) Wild-type embryo showing *msh* nonuniformly expressed in the dorsal third of the neuroectoderm. (B) *ind* marks the intermediate third of the neuroectoderm, and *vnd* expression (C) is seen in the ventral third of the neuroectoderm. To determine if these genes respond to different concentrations of nuclear Dorsal protein, the expression patterns were examined in transgenic embryos with an ectopic anterior–posterior gradients of Dorsal (see text). (D–F) A *bcd-Toll10B-bcd* trangene creates an ectopic domain of *msh* (D) and *ind* (E), and *vnd* (F) are expressed in addition to the endogenous pattern. These ectopic domains extend over the dorsal half of the embryo, but do not extend into the ventral mesoderm. (G–I) A *hsp83-Toll10B-bcd* generates a much broader gradient of nuclear Dorsal along the anterior–posterior axis. When crossed into a *gastrulation defective (gd)* mutant background, this transgene represents the only source of dorsal–ventral positional information. This trangene creates, ectopic stripes of *msh* (G), *ind* (H), and *vnd* (I) at distinct positions along the anterior–posterior axis, suggesting that these genes respond to distinct Dorsal concentrations.

the dorsal ectoderm (St Johnston and Gelbart, 1987). Sog is secreted from the lateral neuroectoderm, forming an extracellular gradient of Sog protein. This Sog protein gradient forms a reciprocal Dpp activity gradient as Sog binds Dpp to inhibit signaling (Ashe and Levine, 1999; Marques et al., 1997).

The mesoderm is initially subdivided into ventral and lateral lineages; ventral regions form somatic muscles (Azpiazu and Frasch, 1993; Bate and Rushton, 1993; Dohrmann et al., 1990; Leptin et al., 1992), while lateral regions form visceral mesoderm and internal organs such as the heart and fat body (Azpiazu and Frasch, 1993; Bodmer, 1993; Bodmer et al., 1990). This subdivision depends on three Dorsal target genes, *twist, snail,* and *dpp* (Frasch, 1995; Maggert et al., 1995). *twist* and *snail* are activated in the ventral-most 18–20 cells by high concentrations of the Dorsal gradient. The *snail*-expressing cells invaginate to become mesoderm, forming a monolayer on the internal surface of the ectoderm. Mesodermal cells that migrate dorsally come into contact with *dpp*-expressing ectoderm cells. Dpp signaling then induces the underlying mesoderm cells to express *tinman* and other genes required for the differentiation of lateral cell types (Maggert et al., 1995; Yin and Frasch, 1998).

This study examines the subdivision of the neuroectoderm into four distinct neuronal cell types: the mesectoderm at the ventral midline, as well as medial, intermediate, and lateral neuroblasts within the ventral nerve cord. The initial subdivision of the neuroectoderm depends on regulatory genes that respond to distinct thresholds of the Dorsal gradient (Rusch and Levine, 1996; Stathopoulos and Levine, 2002). The mesectoderm arises from two single-cell rows

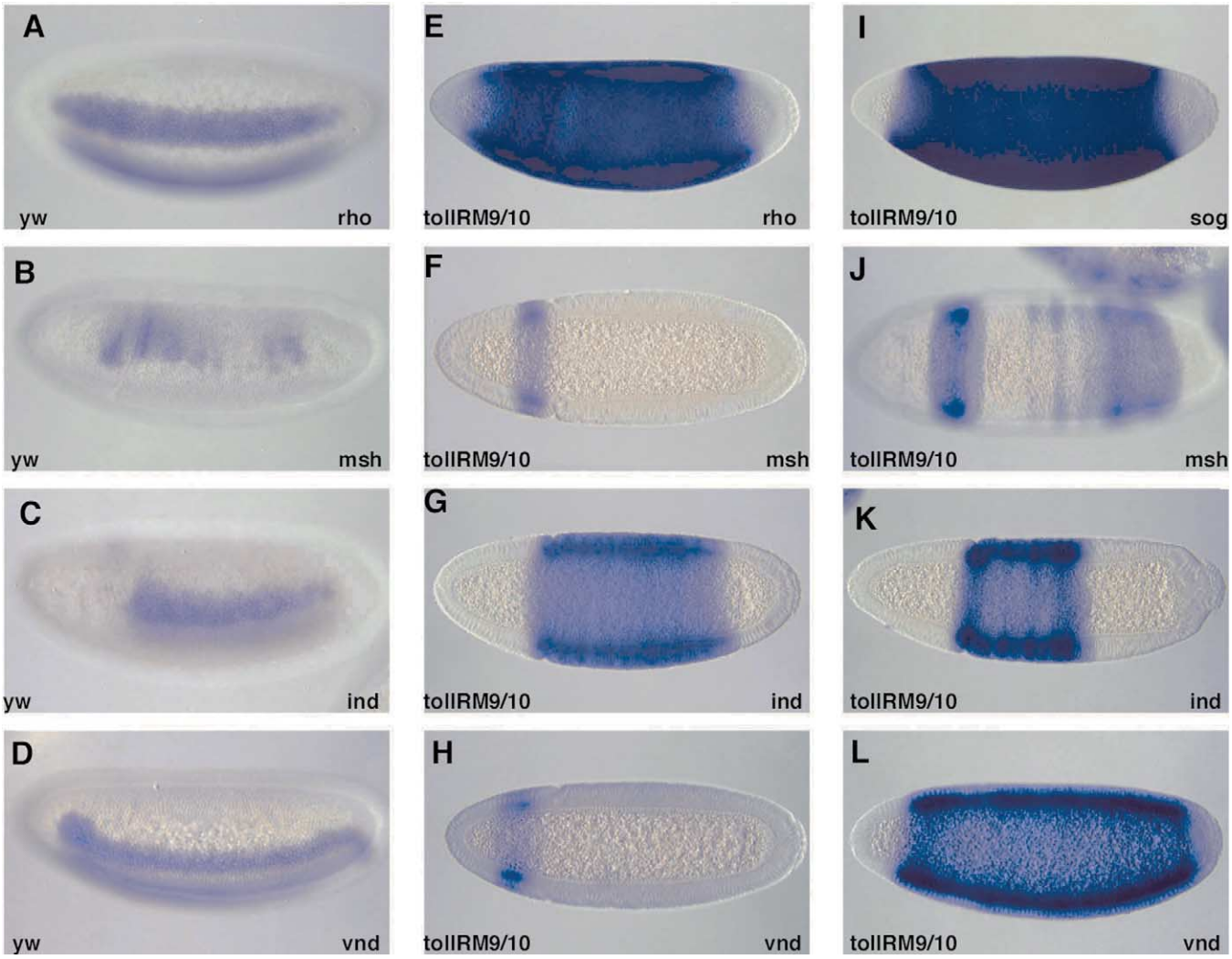

Fig. 2. Expression patterns of *rhomboid (rho), sog, msh, ind,* and *vnd* in wild-type (A–D) and $Toll^{rm9}/Toll^{rm10}$ embryos (E–L). $Toll^{rm9}/Toll^{rm10}$ embryos have low levels of Dorsal everywhere. These embryos express neuroectoderm genes such as *rho* (E, compare with A) and *sog* (I) around the entire circumference of the embryo, transforming the embryo into neuroectoderm. In a great majority of the embryos, the low levels of nuclear Dorsal are sufficient to turn on *ind* (G, compare with C) in the center of the embryo, but not *vnd* (H, compare with D) or *msh* (F, compare with B). In a few embryos, *ind* shows narrower expression in the central regions (K, compare with G), and these embryos show a corresponding increase in *msh* expression (J, compare with F). A small percentage of $Toll^{rm9}/Toll^{rm10}$ embryos have high enough levels to activate *vnd* around the entire dorsal–ventral axis (L, compare with H). This variability is likely a result of nonuniform levels of nuclear Dorsal generated by the partially activated $Toll^{rm9}/Toll^{rm10}$ receptors.

straddling the ventral mesoderm. These cells express the Dorsal target gene *single-minded* (*sim*), a bHLH-PAS protein important in activating mesectoderm-specific target genes (Crews et al., 1988; Nambu et al., 1990, 1991). The *sim* 5′ cis-regulatory region contains several high-affinity Dorsal binding sites, as well as binding sites for the Dorsal target gene *twist* (Kasai et al., 1998). Sim transcription is inhibited in ventral regions by the zinc finger Snail repressor. The dorsal border of the *sim* expression pattern appears to depend on Notch signaling (Cowden and Levine, 2002; Morel et al., 2001; Morel and Schweisguth, 2000).

Three highly conserved homeobox genes, *vnd, ind,* and *msh,* are sequentially expressed in the medial, intermediate, and lateral neuroblasts, respectively (Chu et al., 1998; Cornell and Ohlen, 2000; Cowden and Levine, 2002; D'Alessio and Frasch, 1996; Isshiki et al., 1997; McDonald et al., 1998; Mellerick and Nirenberg, 1995; Weiss et al., 1998). Homologs of these genes are expressed in the same dorsal–ventral positions in the vertebrate neural tube, suggesting that the patterning of the central nervous system (CNS) may be conserved (Chu et al., 1998; Cornell and Ohlen, 2000; D'Alessio and Frasch, 1996; Weiss et al., 1998). In *Drosophila,* the initial *vnd, ind,* and *msh* expression patterns have been suggested to represent distinct threshold readouts of the Dorsal gradient (Chu et al., 1998; Mellerick and Nirenberg, 1995; von Ohlen and Doe, 2000). Indeed, a *vnd* intronic enhancer contains several Dorsal and Twist binding sites (Stathopoulos and Levine, 2002). Moreover, the early

vnd and *ind* expression patterns are absent in embryos lacking Dorsal (Mellerick and Nirenberg, 1995; von Ohlen and Doe, 2000), and ectopic Dorsal results in the misexpression of *vnd* and *ind* (von Ohlen and Doe, 2000). Maintenance of sequential patterns of *vnd, ind,* and *msh* expression has been proposed to depend on cross-regulatory interactions (Cornell and Ohlen, 2000; Mellerick and Nirenberg, 1995; von Ohlen and Doe, 2000). For example, *ind* expression expands ventrally in *vnd* mutants, resulting in a transformation of ventral neuroblasts into intermediate neuroblasts (Weiss et al., 1998). Similarly, *msh* expands ventrally in *ind* mutants to encompass both the dorsal and intermediate columns of the neuroectoderm (Weiss et al., 1998). This study provides additional evidence that the Dorsal gradient establishes distinct *vnd, ind,* and *msh* expression patterns. A constitutively activated form of the *Toll* receptor ($Toll^{108}$) (Schneider et al., 1991) was used to create an ectopic Dorsal gradient along the anterior–posterior axis. The ectopic gradient activated *vnd, ind,* and *msh* in distinct positions along the anterior–posterior axis, suggesting that different concentrations of nuclear Dorsal protein activate these genes. Further evidence for distinct threshold responses was obtained by analyzing mutant embryos containing ubiquitously low levels of nuclear Dorsal sufficient to activate *ind* expression, but not *vnd.* In addition, cross-regulatory interactions are shown to conform to a ventral dominance model, whereby genes expressed in ventral regions, such as *vnd,* repress all genes expressed in more dorsal regions, such as *ind* and *msh.* This ventral dominance extends to regulatory genes expressed in the mesoderm and mesectoderm. In particular, Snail is shown to repress the expression of *sim, vnd, ind,* and *msh.* Repression of *ind* by Vnd is direct and appears to depend on the Groucho corepressor protein. We discuss dorsal–ventral patterning of the neuroectoderm with respect to a "ventral dominance" model, reminiscent but mechanistically distinct from the posterior prevalence model governing Hox gene expression across the anterior–posterior axis.

Materials and methods

In situ hybridization

Embryos were collected, fixed, and then hybridized with dioxygenin-UTP labeled antisense RNA probes as previously described (Jiang et al., 1991). The *snail* and *sim* cDNAs used to produce these probes have been previously described (Ip et al., 1992b; Kosman et al., 1991). The *ind* cDNA used to generate antisense RNA probe was a gift from J. Weiss. The *vnd* cDNA used to generate antisense RNA probe was a gift from T. von Ohlen. The *msh* EST used to generate antisense RNA probe was ordered from Research Genetics.

P-element transformation vectors

The construction of the *bcd-*$Toll^{108}$*-bcd* 3′UTR has been described previously (Huang et al., 1997). For the construction of the *Krüppel-sim, Krüppel-vnd, Krüppel-ind,* and *Krüppel-msh* transformation vectors, the cDNAs were placed under the control of the *Krüppel* enhancer by cloning them into an unique AscI site of a modified pCasPer injection vector. The injection vector contains two tandem copies of a 700 bp *Krüppel* enhancer upstream of a frt-stop-frt cassette. These constructs were then injected into *yw* embryos as previously described (Nibu et al., 1998a). The construction of the *stripe2-snail* has been previously described (Cowden and Levine, 2002). To remove the frt-stop-frt cassette, transgenic females were mated with males homozygous for the yeast Flp recombinase under the control of a sperm-specific tubulin promoter (Wu et al., 1998). F1 males containing both the transgene and the Flp recombinase were selected for subsequent crosses. The F2 progeny derived from these males will have ectopic expression due to the rearrangement of the frt-stop-frt cassette.

For the construction of *IAB5-indvbs-evelacZ,* the 220 bp fragment located 3′ of *ind* (indvbs) was PCR amplified from genomic DNA using the following primers:

indvbsFOR: 5′-ATCGGAATTCCGGATCGAAGAGCCCACGCAACACA-3′
indvbsREV: 5′-ATCGAAGCTTCCCCGGATCTCATCCCGATCGTTATC-3′.

The PCR product was then subcloned downstream of the IAB5 enhancer into a modified pBluescript vector using the *Eco*RI and *Hin*dIII restriction sites introduced by the primers. The modified Bluescript vector contains two *Asc*I sites flanking the IAB5-indvbs insert. The IAB5-indvbs insert was then cloned into a unique *Asc*I site of a modified pCasPer injection vector containing the *even-skipped (eve)* promoter driving *lac*Z expression.

For the construction of *IAB5-mutindvbs-evelacZ,* PCR mutagenesis was used to eliminate the three Vnd binding sites from 5′-CAAGTG-3′ to 5′-CCCGGG-3′ (indmutvbs). The following mutagenic PCR primers were used for the mutagenesis, using the pBSKAscI-*IAB5-indvbs* subclone as a template:

mutvbsFOR: 5′-GAGCTCTTCAGCTCCCGGGGAAGAGGCGCACCCGGGAGCAAGG-3′
mutvbsREV: 5′-CTTGCTCCCGGGTGCGCCTCTTCCCCGGGAGCTGAAGAGC-3′,

The first PCR reaction used indvbsFOR and mutvbsREV as one primer pair and indvbsREV and mutvbsFOR as the other primer pair. The products of these two PCR reactions were then pooled and used as template for a full-length PCR reaction with indvbsFOR and indvbsREV as primer pairs. This mutagenized PCR product was then subcloned into the modified Bluescript vector containing the IAB5 enhancer and the IAB5-mutindvbs insert was cloned into the

pCasPer-evelacZ injection construct as described for *IAB5-indvbs-evelacZ.* Sequencing confirmed the presence of the three mutagenized Vnd binding sites.

The Kreggy misexpression vector uses the *Krüppel* enhancer to drive expression of a protein fused in frame to the GAL4 DNA binding domain (amino acids 1–94). Its construction has been previously described (Nibu et al., 1998b). The Kreggy-*vnd1-543* was PCR amplified using the *vnd* cDNA as template. All of the forward primers introduced a *BsiWI* site at the 5′ end, while the reverse primers had a stop codon followed by an *Xba*I site at the 3′ end. The PCR products were then cloned into the *Acc65I* and *Xba*I sites of the Kreggy injection construct, fusing each protein domain in frame to the GAL4 DNA binding domain. The primer pairs are

vnd342for: 5′-ATCGCGTACGATGACCACGTCGGCGTCCTTG-3′
vnd1971rev: 5′-ATCGTCTAGATTACTTATTTGGCAGACCGTCGGA-3′

These PCR products were also subcloned using the Promega T/A cloning kit in order to make antisense RNA probes.

To make the Kreggy-*vnd1-543ΔVEH1* constructs, PCR based mutagenesis was used to change the FxIxxIL motif to AxAxxAA. A nested PCR strategy was employed, using the two mutagenic primers shown below and the vnd342for and vnd 1971rev primers used from the nonmutagenied Kreggy constructs

mutVEH1for: 5′-TCTGGCGCCCATGCCTCGGACGCCGCCAATTTGGAGGGC-3′
mutVEH1rev: 5′-CAAATTGGCGGCGTCCGAGGCATGGGCGCCAGAGCGTTG-3′,

For the first PCR reactions, mutVEH1 for and vnd1971rev were the first primer pairs, while vnd342 for and mutVEH1rev were the second primer pairs. The PCR products from these two reactions were pooled and used as template for a PCR reaction using vnd342for and vnd1971rev as primers. This PCR product was then cloned into the Acc65I and XbaI sites of the Kreggy vector using the BsiWI and XbaI sites introduced by the primers. Sequencing confirmed the mutagenesis of the FxIxxIL domain to AxAxxAA.

GST constructs and GST pulldown reactions

The VEH1 and IEH1 GST constructs were built using a modified pGEX 5X-3 vector. This GST vector was modified to include a *Kpn*I and an *Xba*I site in the multiple cloning site. The VEH1 and IEH1 and 10 amino acids on either side of the 23 amino acid consensus sequence domains were PCR amplified using the *vnd* and *ind* cDNAs as templates using the primers shown below. As the IEH1 domain is at the very N-terminus, only 33 amino acids were amplified. These primers introduced a *Kpn*I site at the 5′ end and a stop codon followed by an *Xba*I site at the 3′ end. Once cloned, the GST fusion proteins and GST pulldown reactions were performed as previously described:

GST-VEH1
Kpn1VEH1: 5′-ATCGGGTACCCCTGGCTTATTCGATGCAAAG-3′
Xba1STVEH1: 5′-ATCGTCTAGACTAGCCATGGTGGGCGGCAGCAGC-3′
GST-IEH1
Kpn1IEH1: 5′-ATCGGGTACCATGTCGCGTTCATTTTTGATG-3′
Xba1STIEH1: 5′-ATCGTCTAGACTATGTAGGACTTCCTACTGG-3′.

To mutate the conserved phenylalanine residue in the VEH1 domain, a nested PCR strategy was used. Using the *vnd* cDNA as template for the first PCR reactions, Kpn1VEH1 and VEH1-Frev were one pair of primers and VEH1-Ffor and Xba1STVEH1 were the other set of primers. The products of these two PCR reactions were then combined and used as template for another PCR reaction using Kpn1VEH1 and Xba1STVEH1 as primers. This PCR product was then cloned into the modified pGEX-5X-3 vector accordingly. A mutagenic primer introduced the phenylalanine-to-alanine mutation in the IEH1 domain, such that using the *ind* cDNA as template, Kpn1IEH1-F and Xba1STIEH1 were the primer pairs. This PCR product was then cloned into the *Kpn*I and *Xba*I sites of the modified pGEX5X-3 vector. The mutagenic primers are given below. Sequencing confirmed the mutagenesis.

GST-VEH1-F
VEH1-Ffor: 5′-GTCCCAACGCTCTGGCGCCCATATATCGGAC-3′
VEH1-Frev: 5′-GTCCGATATATGGGCGCCAGAGCGTTGGGAC-3′
GST-IEH1-F
Kpn1IEH1-F: 5′-ATCGGGTACCATGTCGCGTTCAGCTTTGATGGAT-3′.

Fly strains

The *Toll^{rm9}* and *Toll^{rm10}* mutations generate low, ubiquitous levels of nuclear Dorsal transport in affected embryos (Anderson et al., 1985). Generation of *Toll^{rm9}/Toll^{rm10}* embryos has been described previously (Cowden and Levine, 2002). The modified NEE-*lac*Z reporter line (G18) crossed to the Kreggy-*vnd1-543* and Kreggy-*vnd1-543ΔVEH1* driver lines in the Kreggy repression assay has been described elsewhere (Nibu et al., 1998b). *yw* embryos were used as wild-type for all experiments. *Krüppel-GAL4* lines were a gift from P. Leopold. All crosses and collections were carried out at 25°C.

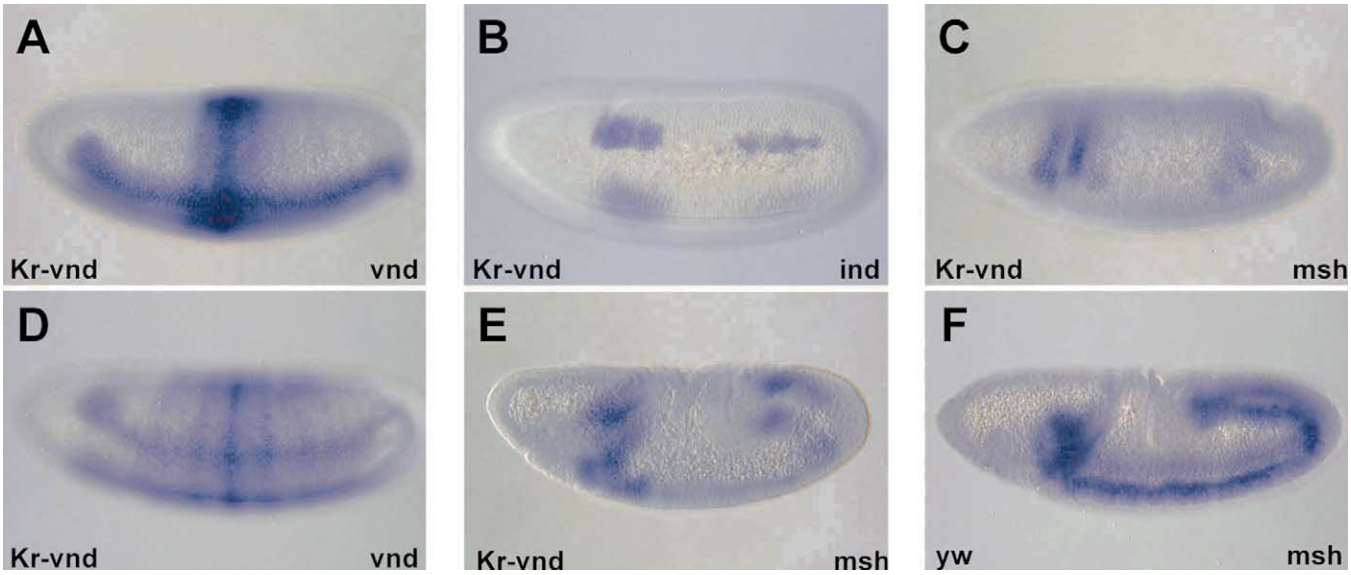

Fig. 3. Ectopic *vnd* represses both *ind* and *msh.* All embryos are lateral views shown with anterior to the left and dorsal to the top. (A, D) *Krüppel-vnd* transgenic embryos hybridized with dioxygenin labeled antisense RNA *vnd* probe to show ectopic expression. The early ectopic expression of a broad, central domain (A) is ultimately downregulated into a stripey lateral pattern (D). (B) *ind* expression is repressed by the ectopic *Krüppel-vnd* transgene. (C) *msh* in a *Krüppel-vnd* transgenic embryo. The *msh* pattern is not uniform at this stage, so any repression activity is not determinable. (E–F) *msh* expression pattern during gastrulation of a wild-type (F) or *Krüppel-vnd* transgenic embryo (E). The *msh* pattern is repressed by the ectopic *vnd.*

Results

vnd, ind, and msh respond to different thresholds of the Dorsal gradient

vnd, *ind*, and *msh* are expressed in sequential lateral stripes within the neuroectoderm (Chu et al., 1998; Cornell and Ohlen, 2000; D'Alessio and Frasch, 1996; Isshiki et al., 1997; McDonald et al., 1998; Mellerick and Nirenberg, 1995; Weiss et al., 1998) (Fig. 1A–C). Due to the patchy onset of *msh* expression (Figs. 1A and 2B), it is unlikely that this gene represents a direct Dorsal target; uniform expression is not seen until the onset of germband elongation (Fig. 3F). Furthermore, *dorsal; dpp* double mutants still express *msh*, suggesting that Dorsal indirectly regulates *msh* expression by repressing Dpp signaling (von Ohlen and Doe, 2000). However, previous studies suggest that the Dorsal gradient might play a direct role in the *vnd* and *ind* expression patterns (Mellerick and Nirenberg, 1995; von Ohlen and Doe, 2000).

As the *vnd* and *ind* expression patterns have different dorsal borders, it is possible that these genes represent distinct thresholds of the Dorsal gradient. Intermediate levels of nuclear Dorsal might be required to activate *vnd* expression, whereas lower levels might be sufficient to activate ind expression. The ability of the Dorsal gradient to establish distinct patterning thresholds in the neuroectoderm was first analyzed in transgenic embryos containing an ectopic anterior–posterior Dorsal gradient (Fig. 1D–F). A constitutively activated Toll receptor, $Toll^{108}$, was placed under the control of the *bicoid* promoter, and localized using the *bicoid* 3′UTR. As a result, the maternally expressed $Toll^{108}$ RNA is localized to the anterior pole and generates an ectopic anterior–posterior Dorsal gradient (Huang et al., 1997). This gradient induces ectopic stripes of *vnd* and *ind* expression (Fig. 1E and F), and augments anterior expression of *msh* (Fig. 1D). All three ectopic patterns extend into the dorsal ectoderm, but are excluded from the ventral mesoderm. The ectopic staining patterns are superimposed on the normal lateral stripes of expression regulated by the endogenous Dorsal gradient. Additional evidence that *vnd, ind,* and *msh* are regulated by different Dorsal concentrations was obtained by analyzing transgenic embryos that lack the endogenous Dorsal gradient (Fig. 1G–I). These embryos contain an *hsp83-*$Toll^{10B}$*-bcd 3′ UTR* transgene that expresses $Toll^{10B}$ RNA at higher levels than the *bcd-*$Toll^{10B}$*-bcd 3′UTR* transgene used in the preceding experiments. The *hsp83-*$Toll^{10B}$*-bcd 3′ UTR* generates the only source of nuclear Dorsal in *gastrulation defective (gd)* embryos (Konrad et al., 1988), creating a broad anterior–posterior Dorsal gradient encompassing the entire length of the embryo. These mutants exhibit sequential bands of *vnd, ind,* and *msh* expression across the anterior–posterior axis (Fig. 1G–I). The maintenance of these distinct, nonoverlapping expression patterns likely depends on cross-regulatory interactions, as described below.

Further evidence that *vnd, ind,* and *msh* respond to different thresholds of the Dorsal gradient was obtained by analyzing mutant embryos containing ubiquitous, low levels of nuclear Dorsal (Fig. 2). $Toll^{rm9}/Toll^{rm10}$ embryos have enough Dorsal to repress genes expressed in the dorsal ectoderm, such as *dpp,* but insufficient levels to activate mesoderm targets such as *twist* and *snail* (data not shown) (Anderson et al., 1985). As a result, the entire dorsal–ventral

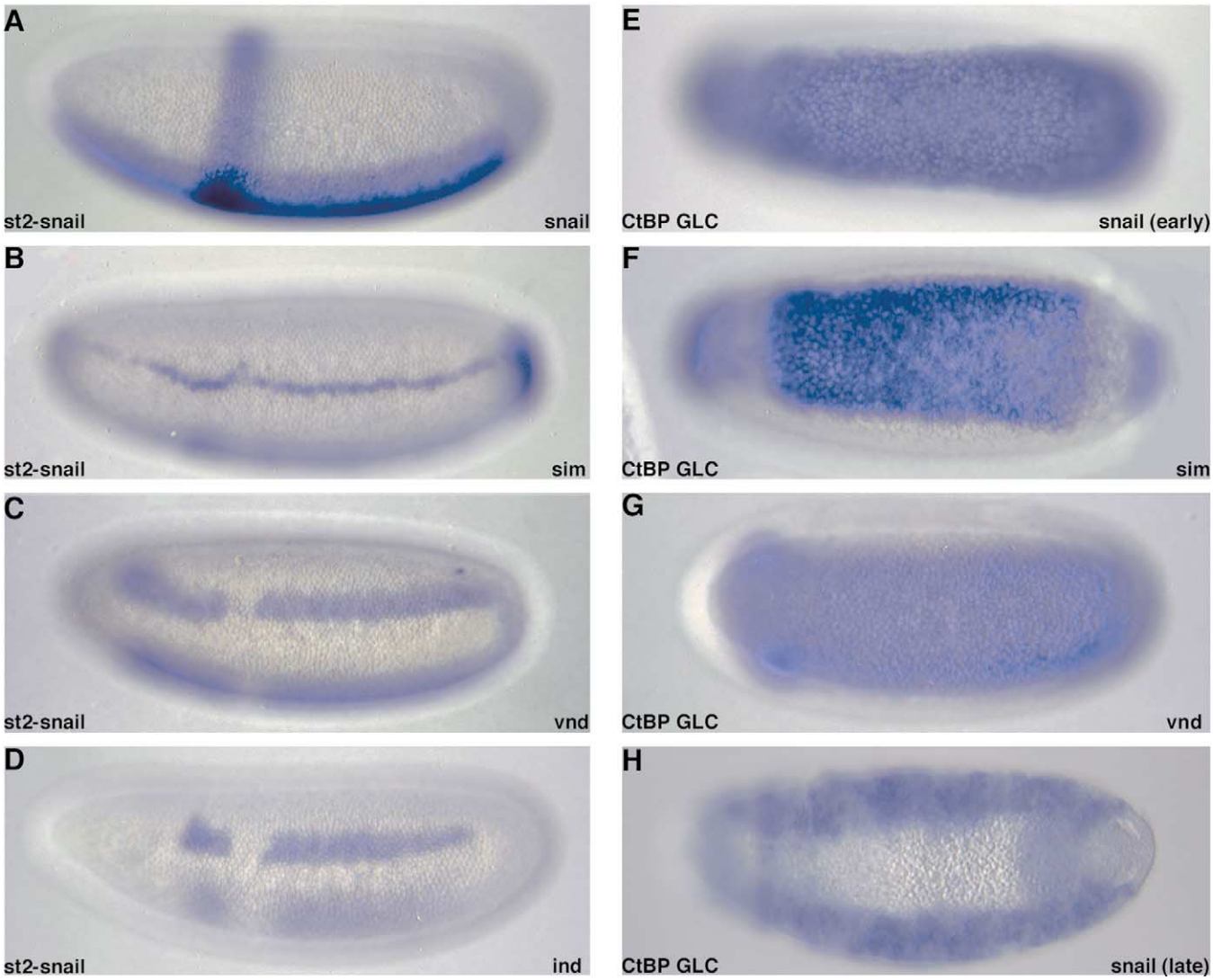

Fig. 4. Evidence for repression cascades in the neuroectoderm and the ventral midline. (A–D) Lateral views with anterior to the left and dorsal to the top of transgenic embryos misexpressing *snail*. (A) A *snail* dioxygenin labeled probe demonstrates that the *eve* stripe 2 enhancer drives ectopic expression of *snail* along the anterior–posterior axis. As a result of this ectopic expression *sim* (B), *vnd* (C), and *ind* (D) all are repressed in the region of the stripe 2-*snail*. (E–H) Ventral views of *CtBP* germ line clone embryos. Though *snail* expression is normal (E), both *sim* (F) and *vnd* (G) show ventral expansion throughout the presumptive mesoderm. However, expression of *snail* (H) shows that the neuroblasts are restricted to the lateral regions of the embryo despite ventral expansion of *vnd*.

axis forms neuroectoderm with uniform expression of *sog* and *rho*, genes normally restricted to lateral stripes of expression (Fig. 2E and I; compare with A). The majority of $Toll^{rm9}/Toll^{rm10}$ embryos exhibit an expanded pattern of *ind* expression, with staining detected throughout central regions (Fig. 2G; compare with C). These embryos exhibit restricted stripes of *vnd* and *msh* expression in anterior regions (Fig. 2F and H; compare with B and D). Some of the mutant embryos exhibit a narrower band of *ind* staining in central regions (Fig. 2K; compare with G); there may be a corresponding expansion of the *msh* staining pattern in these embryos (Fig. 2J; compare with K). Finally, a few embryos show uniform *vnd* expression (Fig. 2L), and it appears that these embryos lack *ind* expression (data not shown). Thus, it would appear that there is some variability in the levels of nuclear Dorsal protein present in $Toll^{rm9}/Toll^{rm10}$ mutants. Most embryos have sufficient levels to activate *ind*, but not *vnd*, while a few embyros have the higher levels needed to activate *vnd*. In addition, in embryos where *vnd* is uniformly expressed, *ind* and *msh* are excluded. This mutual exclusion likely relies on repressive cross-regulatory interactions among these genes as suggested from previous experiments (Chu et al., 1998; McDonald et al., 1998; von Ohlen and Doe, 2000; Weiss et al., 1998).

Vnd represses ind and msh expression

vnd mutants show a ventral expansion of *ind* expression and a corresponding transformation of ventral neuroblasts into intermediate neuroblasts within the ventral nerve cord

(McDonald et al., 1998; Weiss et al., 1998). Misexpression of *vnd* has been shown to repress *ind* in the ventral nerve cord and *msh* in the procephalic neuroectoderm (Chu et al., 1998; McDonald et al., 1998). Similarly, removal of ind^+ gene activity results in a ventral expansion of *msh* into the intermediate column of the neuroectoderm (Weiss et al., 1998). In *vnd; ind* double mutants, *msh* expression expands to encompass the entire neuroectoderm (von Ohlen and Doe, 2000). These results suggested a transcriptional repression model for patterning the neuroectoderm, with Vnd repressing *ind*, and Ind repressing *msh* (Chu et al., 1998; Cornell and Ohlen, 2000; D'Alessio and Frasch, 1996; Isshiki et al., 1997; McDonald et al., 1998; von Ohlen and Doe, 2000; Weiss et al., 1998). This model was tested using a transgenic misexpression system in which a *vnd* cDNA was placed under the control of the *Krüppel* enhancer (Fig. 3).

The *Krüppel* enhancer drives *vnd* expression in a broad central domain prior to gastrulation (Fig. 3A). Ectopic expression is attenuated during gastrulation (Fig. 3D), and the residual pattern exhibits stripes along the anterior–posterior axis. Misexpression of *vnd* causes a gap in the normal *ind* expression pattern (Fig. 3B; compare with Fig. 2C). This repression persists during gastrulation, but is lost by the completion of germband elongation, probably due to transient expression from the *Krüppel-vnd* transgene (data not shown). Because the early expression pattern of *msh* is nonuniform (Figs. 2B and 3C) (Isshiki et al., 1997), it is difficult to determine if ectopic *vnd* expression has any effect on early *msh* expression. However, at later stages, during germband elongation, it is clear that ectopic expression of *vnd* leads to a significant repression in the normal *msh* expression pattern (Fig. 3E; compare with F) (Chu et al., 1998; McDonald et al., 1998). These results confirm that ectopic Vnd represses the expression of both *ind* and *msh* (Chu et al., 1998; McDonald et al., 1998), though these results extend the repressive interactions seen at the ventral nerve cord to the patterning of the lateral neuroectoderm during gastrulation. In *ind* mutants, the *msh* expression pattern is derepressed in intermediate, but not medial, neuroblasts due to the localized expression of Vnd in the medial neuroblasts (von Ohlen and Doe, 2000).

Ventral dominance in the neuroectoderm

While the preceding results confirm the repressive interactions between Vnd, Ind, and Msh, the ability of Vnd to repress *msh* in addition to *ind* raises the possibility that transcriptional repressors expressed in ventral regions of the embryo can inhibit repressors active in more dorsal regions. Support for this hypothesis came from using the *Krüppel* enhancer to misexpress both *ind* and *msh* along the anterior–posterior axis. Ectopic Ind failed to repress *vnd* expression, while ectopic Msh did not repress either *vnd* or *ind* expression (data not shown). To determine if "ventral dominance" was restriced to the neuroectoderm, the mesodermal repressor *snail* was misexpressed in transgenic embryos using the *even-skipped (eve)* stripe 2 enhancer. The *stripe2-snail* transgene creates an ectopic domain of *snail* along the anterior–posterior axis (Fig. 4A). This ectopic expression leads to a gap in the *sim* expression pattern (Fig. 4B). The transgene also causes a gap in the *vnd* pattern (Fig. 4C), confirming the model that Snail excludes *vnd* expression in the ventral mesoderm and restricts expression to the neuroectoderm (Mellerick and Nirenberg, 1995). The *stripe2-snail* transgene also creates a gap in the *ind* pattern (Fig. 4D). These results support the ventral dominance model, whereby repressors located in ventral regions inhibit repressors expressed in more dorsal regions. Consistent with this "directionality" of repression, ectopic expression of Vnd, Ind, or Msh does not repress *snail* (data not shown).

Further support for ventral dominance of the Snail repressor was obtained by analyzing mutant embryos derived from *CtBP* germline clones. *CtBP* is a maternally deposited corepressor protein essential for *snail*-mediated repression (Nibu et al., 1998a,b). Removal of this corepressor results in ventral derepression of *sim* (Fig. 4F) and *vnd* (Fig. 4G) into the presumptive mesoderm due to loss of Snail mediated repression. However, this ventral expansion of *vnd* does not result in a transformation of mesoderm into medial neuroblasts. Instead, the expanded *vnd* pattern is lost at slightly later stages, and expression becomes restricted to lateral regions, similar to the endogenous expression pattern (data not shown). This lateral restriction is consistent with the observation that neuroblasts are formed in lateral regions of $CtBP^-$ mutants, and not in ventral regions that normally form the mesoderm. Neuroblast segregation can be visualized using a *snail* antisense RNA probe, which stains all neuroblasts following gastrulation (Fig. 4H). Sim may be responsible for the late repression of *vnd*, because *vnd* expands into the ventral midline of *sim* mutant embryos (Mellerick and Nirenberg, 1995). Repression of *vnd* by Sim is probably indirect because a *Krüppel-sim* transgene does not alter *vnd* expression in the lateral neuroectoderm (data not shown). Perhaps Sim activates an unknown repressor that ultimately inhibits *vnd* expression in the midline (Estes et al., 2001).

Vnd functions as a sequence-specific transcriptional repressor

It is conceivable that the cross-regulatory interactions among the Snail, Vnd, Ind, and Msh repressors are indirect. For example, perhaps Vnd activates an unknown repressor, which in turn inhibits the expression of *ind* and *msh* in medial neuroblasts. Several experiments were done to determine whether Vnd functions as a transcriptional repressor. The first examined whether Vnd binding sites mediate activation or repression in transgenic embryos.

The IAB5 enhancer drives the expression of a *lacZ* reporter gene in a series of three adjacent bands in the presumptive abdomen of cellularizing embryos (Fig. 5A).

This staining pattern is maintained through gastrulation and germ band elongation (Fig. 5D). Vnd binding sites were introduced into this IAB5-*lacZ* transgene by inserting a 220 bp genomic DNA fragment between the IAB5 enhancer and *lacZ* reporter. This genomic fragment is located 3′ of the *ind* gene and contains three Vnd binding sites (Weiss et al., 1998). Insertion of this fragment caused a ventrolateral gap in the IAB5-*lacZ* staining pattern (Fig. 5B; compare with A). This gap coincides with the endogenous *vnd* expression pattern and is maintained during germ band elongation (Fig. 5E; compare with D). At this stage, there is a clear loss of *lacZ* expression in medial regions of the developing ventral nerve cord. The importance of the Vnd binding sites in mediating this repression was examined by mutagenizing all three sites within the 220 bp DNA fragment. Each site was converted from the 5′-CAAGTG-3′ consensus (Harvey, 1996) to 5′-CCCGGG-3′. The mutagenized IAB5-*lacZ* transgene exhibits expanded expression in medial regions of the presumptive nerve cord (Fig. 5F; compare with E). This observation suggests that Vnd functions as a sequence-specific transcriptional repressor.

Further evidence that Vnd is a repressor was obtained using an in vivo repression assay in transgenic embryos (Fig. 6). The N-terminal region of Vnd contains a putative eh1 Groucho-interaction motif, FxIxxIL (Fig. 6A) (Smith and Jaynes, 1996). This eh1 motif is present in two known transcriptional repressors, Engrailed and Goosecoid (Jimenez et al., 1999; Smith and Jaynes, 1996). It is also found in the Ind and Msh proteins (Fig. 6A) (Smith and Jaynes, 1996). GST pull-down assays suggest that this motif mediates interaction between Vnd and Groucho (Fig. 6B). A GST-VEH1 fusion protein containing amino acid residues 183 to 226 from Vnd binds S^{35}-labeled Groucho protein produced via in vitro translation (see arrow, Fig. 6B). This binding is lost when the GST-Vnd fusion protein is mutagenized to replace the phenylalanine in the FxIxxIL motif with an alanine (GST-VEH1-F; Fig. 6B). Various positive and negative controls were included in these experiments. For example, Groucho does not bind a GST-Ind fusion protein containing the Ind homeodomain. Weak binding is observed with a GST-Eve fusion protein containing the FKPY Groucho-interaction motif (Fig. 6B) (Kobayashi et al., 2001; Zhang et al., 2001).

A *Gal4-Vnd* fusion gene containing the Gal4 DNA binding domain and the N-terminal 543 codons of Vnd was placed under the control of the *Krüppel* 5′ regulatory region. The resulting fusion gene is expressed in central regions of cellularizing embryos (Fig. 6C). Similar levels of expression were obtained with a mutagenized version of the fusion gene that contains multiple alanine substitutions in the FxIxxIL motif (Fig. 6E). The regulatory activities of the two Gal4-Vnd fusion proteins were monitored with a *lac*Z reporter gene that contains a modified version of the *rhomboid* NEE lateral stripe enhancer. The modified NEE enhancer contains three Gal4 binding sites (UAS) and lacks *Snail* repressor sites. The reporter gene is expressed in ventral regions, including the mesoderm and portions of the lateral neuroectoderm (e.g., Fig. 6F).

The unmutagenized Gal4-Vnd fusion protein containing an intact FxIxxIL motif attenuates expression of the *NEE-lacZ* reporter gene (arrowhead, Fig. 6D). This result suggests that the fusion protein binds UAS sites in the modified NEE enhancer and mediates transcriptional repression, either by direct repression of the core promoter, or quenching Dorsal and other activators within the NEE. In contrast, the mutagenized Gal4-Vnd fusion protein (ΔVEH1) fails to repress expression from the *lac*Z reporter gene (Fig. 6F). This result suggests that the FxIxxIL motif is essential for the repression activity of the normal Gal4-Vnd fusion protein. Altogether, these experiments, along with the analysis of Vnd binding sites, suggest that Vnd functions as a sequence-specific transcriptional repressor that might recruit the Groucho corepressor protein.

Discussion

This study provides evidence that the Dorsal gradient directly subdivides the neuroectoderm into separate dorsal–ventral compartments through the differential regulation of three conserved homeobox genes, *vnd, ind,* and *msh.* Maintenance of sequential patterns of gene expression depends on cross-regulatory interactions, whereby repressors expressed in ventral regions inhibit repressors active in more dorsal regions. This ventral dominance is evocative of the posterior prevalence phenomenon that governs sequential patterns of Hox gene expression across the anterior–posterior axis of metazoan embryos. At least one of the cross-regulatory interactions is direct and evidence was presented that Vnd functions as a sequence-specific transcriptional repressor. We discuss the conservation of the Vnd-Ind-Msh repression cassette in patterning the vertebrate neural tube.

Dorsal prepatterns the neuroectoderm

The Dorsal gradient establishes at least three thresholds of gene expression across the dorsal–ventral axis of early embryos (Rusch and Levine, 1996; Stathopoulos and Levine, 2002). High concentrations activate target genes such as *twist* and *snail* in ventral regions that form the mesoderm (Ip et al., 1992b). Intermediate concentrations activate the *rhomboid* gene in ventral regions of the neuroectoderm (Ip et al., 1992a). Finally, low levels of the gradient activate the *sog* gene in both ventral and dorsal regions of the neuroectoderm. The same low levels of Dorsal repress target genes important for the differentiation of the dorsal ectoderm, including *dpp, zen,* and *tolloid.*

Mutant embryos lacking Dorsal fail to activate early expression of either *vnd* or *ind* (Mellerick and Nirenberg, 1995; von Ohlen and Doe, 2000). Conversely, ectopic Dorsal activity leads to a corresponding dorsal shift in the *vnd* and *ind* expression patterns (von Ohlen and Doe, 2000). The

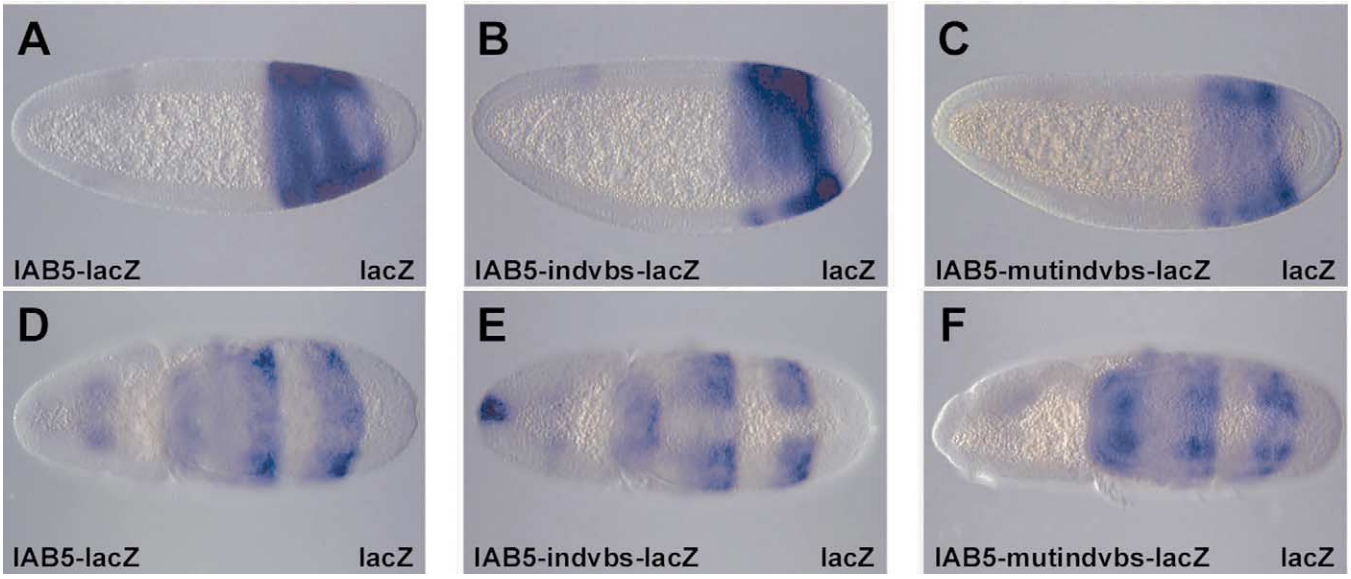

Fig. 5. Addition of Vnd binding sites mediates repression. Lateral views (A–C) of pregastrula embryo and ventral views (D–F) of germ-band elongation embryos hybridized with dioxygenin labelled *lacZ* probes. Embryos are oriented with anterior to the left and lateral views have dorsal toward the top. (A, D) The IAB5 enhancer alone drives expression of *lacZ* in a broad posterior band prior to gastrulation (A) and in three stripes at germ band elongation (D). (B, E) Addition of Vnd binding sites mediates repression of the posterior band (B) and the three stripes at germ band elongation (E). (C, F) Mutating these binding sites eliminates repressive activity in the pregastrula (C) and germ band elongation (F) embryos.

lateral stripes of *vnd* expression encompass ventral regions of the neuroectoderm, similar to the *rhomboid (rho)* pattern. *rho* is a direct Dorsal target gene (Ip et al., 1992a) that is expressed in the neuroectoderm (Fig. 2A) and encodes a membrane-associated protease that processes the EGFR ligand *spitz* (Bang and Kintner, 2000). Like *rho, vnd* appears to be a direct target of the Dorsal gradient, as an intronic enhancer containing clustered Dorsal and Twist binding sites directs lateral stripes of expression in transgenic embryos (Stathopoulos and Levine, 2002). The *ind* lateral stripes appear to straddle the region between the *vnd/rhomboid* ventrolateral stripes and the broad *sog* lateral stripes, and previous studies suggest that *ind* may be regulated in a different manner from *vnd.* The regulation of *ind* relies on both the Dorsal gradient and the EGF signaling pathway (Skeath, 1998; von Ohlen and Doe, 2000). Removal of either Dorsal or the EGF receptor results in the loss of *ind* expression from the neuroectoderm (von Ohlen and Doe, 2000). It is unclear whether Dorsal directly activates *ind* or simply establishes a domain of EGF signaling through the regulation of *rhomboid (rho).* However, given the early onset of *ind* expression and the misexpression of *ind* by ectopic Dorsal (von Ohlen and Doe, 2000), it is likely that Dorsal is essential for its regulation. Consistent with the possibility that early *ind* expression pattern might reflect a threshold readout of the Dorsal gradient is the finding that the low levels of Dorsal present in $Toll^{rm9}/Toll^{rm10}$ embryos are sufficient to activate *ind,* but not *msh.* Moreover, the *ind* lateral stripes do not extend beyond the *sog* expression pattern, which is known to be directly activated by vanishingly low levels of the Dorsal gradient. Finally, a 3′ *ind* enhancer that encompasses the three Vnd binding sites used in this study (Fig. 5) contains optimal Dorsal and Twist binding sites, suggesting that it is directly regulated by the Dorsal and Twist gradients (A. Stathopoulos, unpublished results).

The initial compartmentalization of the neuroectoderm appears to depend on threshold readouts of the Dorsal gradient. This strategy is different from the subdivision of the other two primary embryonic tissues, the mesoderm and dorsal ectoderm. Patterning the mesoderm depends on interactions between *twist* and *dpp* (Frasch, 1995; Maggert et al., 1995). The Snail repressor establishes the limits of mesoderm invagination, while the localized expression of Dpp restricts induction of the lateral mesoderm to dorsal–lateral regions (Maggert et al., 1995). Similarly, subdivision of the dorsal ectoderm depends on the differential regulation of the Dorsal target genes *sog* and *dpp* (Francois et al., 1994; Zusman et al., 1988). Both genes respond to the same low levels of the Dorsal gradient, but *sog* is activated by Dorsal, while *dpp* is repressed (St Johnston and Gelbart, 1987). Subsequent protein–protein interactions between Sog and Dpp establish a broad Dpp signaling gradient in the dorsal ectoderm (Ashe and Levine, 1999; Marques et al., 1997).

A repression cascade patterns the neuroectoderm

Transcriptional repression of *ind* by Vnd was predicted from previous genetic studies but lateral repression of *msh* was somewhat unexpected (Chu et al., 1998; McDonald et al., 1998; Weiss et al., 1998). Previous studies have shown

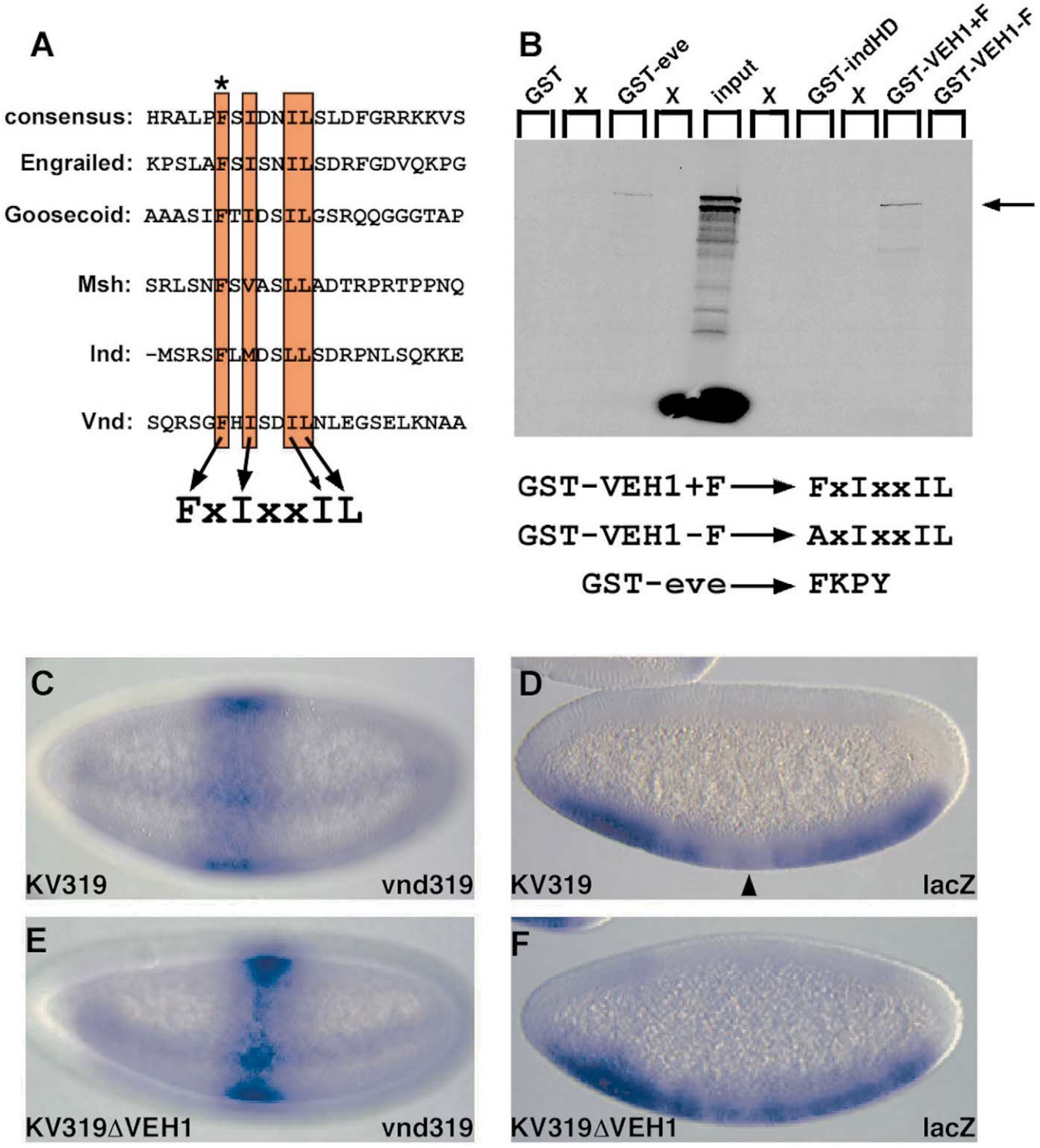

Fig. 6. *vnd*, *ind*, and *msh* all contain a putative eh1 domain. (A) Alignments of the *vnd*, *ind*, and *msh* putative eh1 domains against the 23 amino acid eh1 consensus as well as the eh1 domains from *engrailed* and *goosecoid.* The boxes indicate the highly conserved FxIxxIL motif and the asterix marks the phenyalanine residue known to be important in interacting with Groucho. (B) GST pulldowns with S^{35}-labeled Groucho. GST and GST-indHD, with the homeodomain from *ind* fused to GST, were used as negative controls. GST-*even-skipped* (GST-eve) was used as a positive control for *groucho* interaction as *eve* is known to interact with *groucho* through the FKPY motif. The VEH1 domain interacts with *groucho* when fused to GST, but mutating the conserved phenylalanine residue to alanine abolishes the in vitro interaction. Input represents 1/10th of S^{35}-labelled *groucho.* (C) The Kreggy misexpression construct (KVnd1-543) drives ectopic expression of the N-terminal region of Vnd, as assayed with a dioxygenin-labeled probe to *vnd* (D) The ectopic N-terminal domain of Vnd represses the *lacZ* reporter gene, as indicated by the arrowhead. (E, F) Kreggy misexpression construct (KVnd1-543ΔVEH1) drives expression of N terminal region of Vnd with the VEH1 domain mutated from FxIxxIL to AxAxxAA (see Materials and Methods). While the Kreggy vector drives expression of the mutant Vnd domain (E), only weak repression of the *lacZ* reporter gene is detected (F).

that ectopic Vnd represses *msh* expression in the procephalic neuroectoderm (Chu et al., 1998), where the *vnd* and *msh* expression patterns overlap. This result was extended in the present study using a *Krüppel-vnd* transgene. It would appear that Vnd represes both *ind* and *msh* to specify medial neuroblasts (Chu et al., 1998; McDonald et al., 1998; Weiss et al., 1998). A similar result was seen using the *eve* stripe 2 enhancer to misexpress *snail.* Previous studies have shown that Snail acts as a transcriptional repressor to create the boundary between mesoderm and neuroectoderm (Kosman et al., 1991). As expected, ectopic *snail* repressed *vnd* expression but surprisingly, *ind* was also repressed. These results suggest that the Dorsal gradient separates domains along the dorsal–ventral axis by activating a series of localized transcriptional repressors (Fig. 7A). According to this model, repressors located in ventral regions selectively repress those located more dorsally, while dorsal repressors do not inhibit ventral repressors. For example, ectopic Vnd represses *ind* but not *snail* (Fig. 7B), while ectopic Ind fails to repress *vnd* or *snail* (data not shown). According to this model, ectopic Ind should repress *msh* expression. However, because none of the transgenic *Krüppel-ind* lines persisted until germband elongation when *msh* expression is uniform, it was not possible to determine if ectopic Ind repressed *msh.* Similarly, while ectopic Msh failed to repress *snail, vnd,* or *ind* expression (data not shown), the lack of early target genes that are regulated by Msh prevents any definitive conclusions regarding its role as a transcriptional repressor. Both Ind and Msh contain putative eh1 domains, suggesting that they may function as Groucho dependent repressors and previous work supports such a role for Ind and Msh in the ventral nerve cord (Isshiki et al., 1997; Weiss et al., 1998).

Several lines of evidence suggest that Vnd functions as a Groucho-dependent sequence-specific transcriptional repressor. First, misexpression of Vnd leads to the repression of *ind* and *msh* (Chu et al., 1998; McDonald et al., 1998). Second, Vnd binding sites located 3′ of the *ind* transcription unit (Weiss et al., 1998) mediate transcriptional repression in vivo when placed next to the heterologous IAB5 enhancer. Third, the N-terminal region of Vnd contains a conserved Groucho interaction motif, FxIxxIL, and this motif is important for Vnd–Groucho interactions in vitro (Jimenez et al., 1999; Smith and Jaynes, 1996). Fourth, mutations in this motif abrogate the repression activity of an otherwise normal Gal4-Vnd fusion protein in transgenic embryos.

Transcriptional repression and the conservation of dorsal–ventral patterning

Interactions among *vnd, ind,* and *msh* have been shown to play an important role in maintaining the neuroectodermal subdivisions established by the Dorsal gradient. Maintenance of these three regions is crucial for the proper patterning of the ventral nerve cord following gastrulation (Chu et al., 1998; McDonald et al., 1998; Weiss et al., 1998). Embryos mutant for *vnd* show a ventral expansion of *ind* and subsequent transformation of medial neuroblasts into intermediate neuroblasts (McDonald et al., 1998; Weiss et al., 1998). Transformation of intermediate neuroblasts into lateral and ventral neuroblasts is seen in *ind* mutant embryos, which exhibit a ventral expansion of *msh* during gastrulation (Weiss et al., 1998). Finally, double mutants for *vnd* and *ind* show an expansion of the *msh* expression pattern throughout the neuroectoderm and the transformation of all neuroblasts into lateral neuroblasts (von Ohlen and Doe, 2000). This genetic evidence suggests that while *vnd, ind,* and *msh* represent distinct threshold responses to the Dorsal gradient, it is transcriptional repression which maintains the proper pattering in the neuroectoderm. The *vnd, ind,* and *msh* expression patterns seen in $Toll^{rm9}/Toll^{rm10}$ are consistent with cross-repressive interactions among these genes. While a majority of these mutant embryos show uniform *ind* expression, a smaller number exhibit nonoverlapping domains of *ind* and *msh* expression in the center of the embryo. A few of the mutants show uniform *vnd* expression, and in this case both *msh* and *ind* are excluded from central regions (data not shown). The simplest explanation of these observations is that the $Toll^{rm9}/Toll^{rm10}$ mutant embryos contain somewhat variable levels of nuclear Dorsal protein. Those containing relatively high levels express *vnd,* which in turn leads to the repression of *ind* and *msh.* In contrast, mutant embryos containing lower levels of nuclear Dorsal express *ind; vnd* is absent due to insufficient levels of Dorsal and *msh* is repressed by Ind.

"Ventral dominance" might govern the patterning of the ventral nerve cord in older embryos, in addition to the prepatterning of the neuroectoderm in pregastrulating embryos. Sim might exclude *vnd, ind,* and *msh* expression in the ventral midline (Estes et al., 2001; Mellerick and Nirenberg, 1995). In embryos lacking maternal CtBP products, Snail fails to act as a repressor (Nibu et al., 1998a, 1998b), allowing the ventral expansion of *sim* and *vnd* into the presumptive mesoderm (Fig. 4F and G). However, *vnd* expression is ultimately lost from ventral regions, while *sim* expression persists. As a result, ventral regions form an expanded mesectoderm, while neuroblasts arise from lateral regions (Fig. 4H). These observations suggest that Sim excludes *vnd* expression from ventral regions in *CtBP* mutants, either directly by acting through a CNS specific enhancer or indirectly by activating an unknown repressor. This putative repressor probably does not rely on the CtBP corepressor, as it is still capable of repressing *vnd* in *CtBP* germ line clones. According to a ventral dominance scenario, the misexpression of this unknown repressor should inhibit the expression of *vnd, ind,* and *msh* in the ventral midline (Fig. 7B). One potential target for the indirect repressor could be the EGF pathway. The ventral midline is a well-characterized source of EGF signaling (Golembo et al., 1996) and both *vnd* and *ind* rely upon EGF signaling for maintenance of expression (Skeath, 1998; von Ohlen and

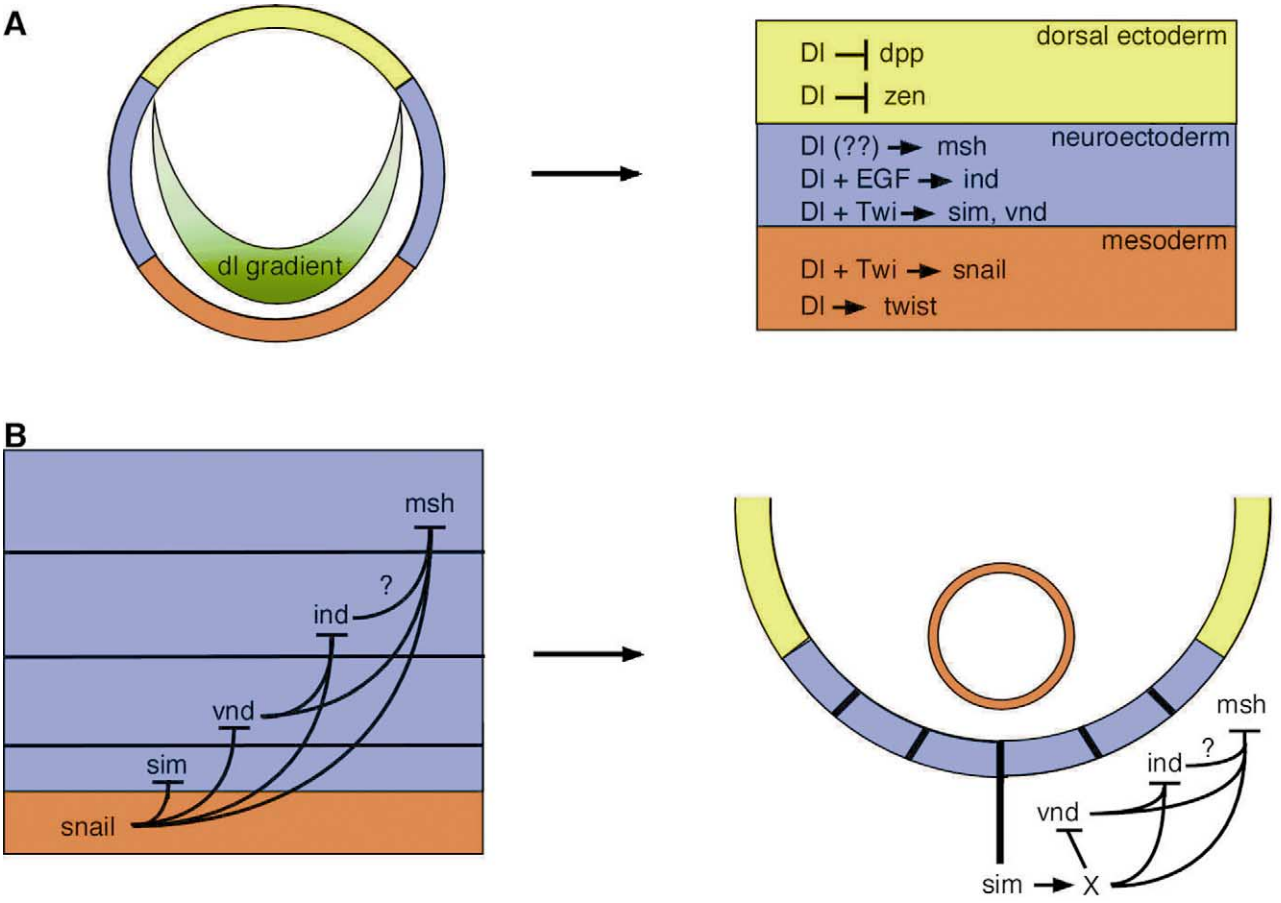

Fig. 7. Summary of ventral dominance model. Red indicates mesoderm, blue indicates neuroecotderm, and yellow indicates dorsal ectoderm. (A) The broad nuclear Dorsal concentration gradient forms the three primary tissues by acting as an activator and a repressor. In the ventral mesoderm, Dorsal activates *twist*, and then Dorsal and Twist activate *snail* expression. In the lateral neuroectoderm, Dorsal acts with Twist to activate both *sim* and *vnd*. Dorsal and the EGF pathway regulate *ind* expression and Dorsal indirectly establishes *msh* expression by repressing Dpp signaling. Both *dpp* and *zen* are restricted to the dorsal ectoderm via repression by Dorsal. (B) Interactions among dorsal target genes maintains the subdivision of the neuroectoderm through transcriptional repression. Those repressors located more ventrally are capable of repressing target genes located more dorsally. For instance, Snail is capable of repressing *sim*, *vnd*, *ind*, and possibly *msh*, while Vnd represses *ind* and *msh* but not *snail*. Following gastrulation, *sim* potentially activates an unknown repressor X to establish a similar repression cascade at the ventral nerve cord. Question marks indicate where the repression activity has not been shown.

Doe, 2000). By eliminating EGF activation, this midline repressor could prevent *vnd* and *ind* expression.

It is conceivable that the ventral dominance model governing cross-regulatory interactions among Vnd, Ind, Msh, Snail, and possibly *sim*, also applies to the patterning of the vertebrate neural tube (Cornell and Ohlen, 2000; Weiss et al., 1998). The vertebrate homolog of *vnd*, *Nkx2.2*, is expressed in ventral regions of the neural tube, while the homologs of *ind (Gsh)* and *msh (Msx)* are expressed in intermediate and dorsal regions, respectively (Cornell and Ohlen, 2000). These neural tube expression patterns match the dorsal-to-ventral positions of *vnd*, *ind*, and *msh* in the ventral nerve cord of *Drosophila*. Furthermore, the vertebrate homolog of Vnd, Nkx2.2, also functions as a Groucho-dependent transcriptional repressor (Muhr et al., 2001). A clear prediction of this study is that the misexpression of Nkx2.2 throughout the vertebrate neural tube should lead to the repression of both *Gsh* and *Msx*. In contrast, the misexpression of Gsh should repress *Msx*, but not *Nkx2.2*. Thus, a cascade of homologous localized transcriptional repressors could subdivide both the vertebrate and invertebrate CNS.

Acknowledgments

The authors would like to thank J. Weiss for providing the *ind* cDNA and *vnd* mutant files, T. v. Ohlen for providing the *vnd* cDNA, and P. Leopold for providing the Kr-GAL4 driver lines. We also thank A. Stathopoulos for sharing unpublished data and S. Beckendorf for helpful advice and suggestions. This work was funded by a grant from the NIH (GM46638).

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